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Tumbleweed (*Salsola*, section *Kali*) species and speciation in California

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Abstract Tumbleweeds (*Salsola* species, section *Kali*) are road side and rangeland pest plants throughout the 48 contiguous states in the US. Three described tumbleweed species and two undescribed *Salsola* taxa occur in California. The known species are Russian thistle, *Salsola tragus*, introduced from Eurasia in the 1800s, Russian barbwire thistle, *S. paulsenii*, which grows in the desert regions of California, and is also native to Eurasia, and the recently identified *S. kali* subspecies *austroafricana*, possibly native to South Africa. Our goals were to investigate karyology, genome size, and molecular genetic affinities of the described species and the other taxa within their ranges in California using recently developed microsatellite loci, dominant nuclear DNA markers (RAPD and ISSR), and DNA sequence data. Chromosome counts and genome size assessments made with flow cytometry were compared. These analyses indicated that one undescribed taxon is a new allopolyploid hybrid between *S. tragus* and *S. kali* subspecies *austroafricana*, and the other undescribed taxon appears to be a complex hybrid involving all three described species. The invasion potentials for the hybrid taxa are unknown. Tumbleweeds are the focus of biological controls efforts but the identification of suitable agents for the hybrid taxa may be problematic because of the large amount of genetic variability encompassed within this evolving *Salsola* complex.

Keywords Allopolyploid speciation · Invasive hybrids · *Salsola tragus*

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Introduction

The expansion of the range for *Salsola tragus* L. has been described as the most rapid outbreak of an introduced species in North America (Rilke 1999), causing economic damage throughout 6.5 million hectares 20 years after its introduction (Shinn 1895). Spread of the pest occurred through windborne tumbling of entire mature plants, transport in animal bedding and litter in rail cars, and through contamination of agricultural seed, with the latter as

the probable means of the initial introduction. After the initial appearance of *S. tragus* in South Dakota in the 1870s, it reached Antelope Valley in southern California by 1885 (Young 1988) and 10 years later appeared in the Central Valley in Bakersfield along railroad tracks "from seed evidently scattered from cars that have come through Nebraska or some other infected section" (G. F. Weeks reported in Shinn 1895). Transport northward over the Tehachapi Mountains from Antelope Valley was also a possibility.

The native range of *S. tragus* is from eastern Russia to southeast Siberia and northeast China extending south to northern Africa, Saudi Arabia, Pakistan and Nepal (Rilke 1999). Farmers from Russia had settled in the area of the initial outbreak in South Dakota, and this coincidence led to one of the common names for this plant, Russian thistle. Shinn (1895) reported the original source of *S. tragus* in the US as impure flax seed brought from Russia in 1873 to Scotland Township, Bon Homme County, South Dakota. The number of introductions of *S. tragus* to North America is not known although it seems probable that there were several. Young (1988) suggested that there might have been a second introduction to South Dakota during the early years of the infestation. Molecular comparison of *S. tragus* from California and Ukraine indicated that plants from the two regions were similar according to isoenzymic, RAPD and ISSR analyses (Ryan et al. 2007) and phosphoenolpyruvate carboxylase intron DNA sequences (Gaskin et al. 2006).

A second tumbleweed species, *Salsola paulsenii* Litv ($2n = 36$; Mosyakin 1996, 2003; Rilke 1999), was common in a disturbed area immediately east of Barstow, California in 1968 (Munz 1968), and widely distributed at low elevations in the Mojave desert (Beatley 1973) but may have been collected from this area as early as 1913. Beatley (1973) suggested that it had been introduced to the deserts between the Death Valley Expedition of 1891 and the 1913 collection. It is now reported in seven western states, and is expected to spread (Mosyakin 2003). According to Rilke (1999), the native range of *S. paulsenii*, comprising two subspecies, is from SW Russia to western China (Xinjiang and Gansu provinces) and southern Mongolia, southern Iran and Afghanistan, and overlapping *S. tragus* to some extent in SW Russia.

In 2000, a molecular genetic study of variation in *S. tragus*, detected another genetic entity, widespread in California and Arizona, which had escaped taxonomic scrutiny to that point (Ryan and Ayres 2000). This cryptic species, initially termed Type B, was subsequently shown to be separable from *S. tragus* by morphological characters (Hrusa, personal communication) and had a chromosome number $2n = 18$ (J. Bailey, personal communication), in distinction to $2n = 36$ for *S. tragus* (Mosyakin 1996, 2003; Rilke 1999). Recently, Type B was found to have the same DNA sequence as *S. kali* subsp. *austroafricana* Aellen from South Africa and Namibia (Gaskin et al. 2006). Rilke (1999) considered *S. kali* subsp. *austroafricana* to be a variety of *S. tragus* although the Eurasian source of these infestations, if the species is indeed introduced to South Africa and not native, is not known. As this species was not recognized as different from *S. tragus* until recently, there is no information on timing, means, or source of the introduction.

A large amount of morphological variability has been noted in *S. tragus* and other species within the tumbleweeds, *Salsola* section *Kali*, in both their native and introduced ranges (Rilke 1999) which has contributed to the confused taxonomic status of these species with numerous synonyms used over the past 150 years. The dearth of reliable taxonomic characters and discontinuities has compounded the problem. Mosyakin (2003) noted the existence of a high level of polymorphism at the morphological level in *S. tragus* in North America and suggested there may be several varieties and perhaps even species present in the US. Additionally, hybridization may have blurred species boundaries.

Hybridization among *Salsola* species in section *Kali* has been postulated for many years, both in the native range and in North America. Rilke (1999) observed that hybrids between *S. tragus* and *S. paulsenii*, comprising all morphological transition forms between the two species, were found in the greater part of the range of *S. paulsenii* in Eurasia and in North America. Beatley (1973) described the presence of what she inferred to be hybrid swarms of *S. paulsenii* and *S. iberica* (*S. tragus* in current nomenclature) showing introgression between the two parental species in the Mojave Desert where populations of the parents were adjacent on altitudinal gradients. Arnold (1972) investigated the taxonomic

status of two postulated *Salsola* hybrids in Utah and adjacent states; one postulated hybrid was termed *S. paulsenii* lax, the other *Salsola* X, both had chromosome number $2n = 54$. The anatomical, morphological, chemical and palynological characters used were not sufficient to provide separation of the two putative hybrids and *S. paulsenii*, leading to the conclusion that they were members of the same taxon. Wilken (1993) observed that the question of hybridization of *S. paulsenii* and *S. tragus* needed further study. Rilke (1999) noted that karyological investigations on putative hybrids within *Salsola* section *Kali* had not been conducted.

In 1998, another taxonomic entity, distinct from *S. tragus* and *S. kali* ssp. *austroafricana* in isoenzyme complement and morphology, was found near Coalinga and termed Type C (Ryan et al. 1999). Another population of Type C was found in Kern County near the city of Bakersfield during an extensive survey undertaken in 2002 (Akers et al. 2002). The survey, the goals of which were to determine the characteristics and distribution of *Salsola* taxa in the Central Valley of California and adjacent regions, found that pure populations of *S. tragus* were frequently found along roadsides throughout the State, while pure populations of *S. kali* ssp. *austroafricana* were common along roadsides in the Valley and adjacent coast mountains south of Los Banos. They found numerous sites in the Central Valley south of the city of Modesto where *S. tragus* and *S. kali* ssp. *austroafricana* formed mixed stands (Fig. 2 in Akers et al. 2002). A detailed taxonomic study from plants collected during the survey indicated the presence of five distinct taxa based on quantifiable morphological characters (Hrusa, unpublished data): (1) *S. tragus*, (2) *S. kali* ssp. *austroafricana*, (3) Type C, (4) *S. paulsenii*, and (5) *S.* lax (*S. paulsenii* lax in Arnold's (1972) terminology). Microsatellite markers have been developed for these *Salsola* taxa and preliminary results confirm the genetic distinctness of each taxa (McGray et al. 2007). We enlarge upon these results in the present paper.

In the 1970s, the U.S. Department of Agriculture introduced two moth species as biological control agents to slow the spread of *S. tragus* in California (Hawkes and Mayfield 1978; Muller et al. 1990). The two species survived and are common on *Salsola*, but had little or no effect on the weed's abundance (Hawkes and Mayfield 1978; Muller et al. 1990). Work continues today in the search for biocontrol agents for this weed (Bruckart et al. 2004; Smith 2005; Sobhian et al. 2003a, b).

For control efforts to be effective it is critical to know exactly what species are present. This is especially true for biological control efforts where agents may not only be species-specific, but may be specific (or even endemic) to the area from which the invading population originated. Further, if hybrids have arisen, they may be even more invasive than the parental species (Ellstrand and Schierenbeck 2000). Given the invasiveness of the three known species and the ongoing search for biological control agents, it is imperative that we know what taxonomic entities of *Salsola* are present in the state. This work was undertaken to use the tools of DNA-based molecular markers and karyological analyses to complement and supplement taxonomic and morphological studies of introduced *Salsola* species and possible hybrids in California.

Materials and methods

DNA—microsatellites, RAPDs, and ISSRs

Plant samples were collected from throughout the Central Valley of California, and from southern California locations; Death Valley in Inyo County, Victorville in San Bernardino County, and San Diego. Four *S. tragus* samples from Turkey were also included. Samples were tentatively identified to taxa by morphology and, in some cases, by isoenzyme or DNA profiles according to Ryan and Ayres (2000) (F. J. Ryan, unpublished results).

DNA was extracted according to the protocols in Ryan and Ayres (2000). Microsatellite primers, amplification conditions, and alleles are described in McGray et al. (2007). Species-specific microsatellite alleles were identified for *S. tragus*, *S. kali* ssp. *austroafricana*, and *S. paulsenii*; we examined Type C and *S.* lax individuals for the presence or absence of these alleles.

RAPD-PCR and ISSR-PCR

For analysis of samples by RAPD-PCR, primers A8, A13, A18, B7, C18, D5, D18, D20, H2, and H8 (Operon Technologies (QIAGEN), Emeryville, CA)

were used. For analysis by ISSR-PCR primers 807 (55°C), 808 (45°C), 810, 812, 825, 826, 835, 840, 841 (45°C), 848, 850 (55°C), 889, and 890 (55°C) (University of British Columbia Kit 800) were used. Reaction conditions were according to the protocols of Ryan and Ayres (2000) except that an Eppendorf Mastercycler Gradient or a Hybaid PCR Express thermocycler was used and the annealing temperatures for ISSR-PCR were 50°C except where noted above in parentheses. Polymorphic bands were generally scored as present or absent for each sample; in some cases, bands of intermediate intensity were scored as 0.5. Euclidean distance coefficients were calculated and then individuals were clustered using UPGMA as implemented with NTSYS 2.1 (Rohlf 2000).

DNA sequencing

Salsola lax samples ($n = 11$) came from seed collected by P. Akers and F. Hrusa from the south end of the Central Valley (Kern County), southern California (San Bernardino County), the east slope of the Sierra Nevada (Inyo and Mono County), and a sample taken further east from Tonopah, NV. Collection information for other species is listed in Gaskin et al. (2006).

Following extraction of DNA by standard methods (Ryan and Ayres 2000), amplification of the intron between the fourth and fifth exon of the low copy PEPC gene utilized the primer pair ppcx4f (5′-ACTCCACAGGATGAGATGAG-3′) and ppcx5r (5′-GCAGCCATCATTCTAGCCAA-3′). Amplification was conducted after a 2 min denaturation at 95°C and consisted of 30 cycles of 95°C (1 min), 52°C (1 min) and 72°C (2 min); followed by 5 min at 32°C. The two PCR products (one band approximately 500 bases in length and the other ca. 400 bases in length) were present in all samples. These bands were separated by electrophoresis on a 2% agarose gel and the shorter band was excised (the identity of the longer band is unknown, and its sequence variation was not useful for this analysis). DNA was purified with the Qiagen QIAquick Gel Extraction Kit. The resultant template was sequenced on a Beckman CEQ 2000XL DNA Analysis System using reagents and protocols supplied by the manufacturer and the same primers mentioned above. Heterozygotic genotypes were cloned and sequenced in an earlier study to determine the haplotypes involved (Gaskin et al. 2006). The earlier study included 88 individuals from four taxa relevant to this study (*S. tragus*, *S. kali* subsp. *austroafricana*, *S. paulsenii*, and *S.* lax). Known haplotypes from the earlier study were used to estimate haplotypes of the 11 heterozygotes in this study. Haplotype sequences were aligned by hand using SE-Al software (Rambaut 1996) and haplotype sequences are available in GenBank (accession numbers are in Gaskin et al. 2006). Haplotypes from this study were combined with those from Gaskin et al. (2006) and arranged manually into a most parsimonious network (Fig. 2).

Determination of chromosome numbers

Seeds of known origin from California were placed on wet sand in 10 cm Petri dishes. Germination was usually extremely rapid, and seedlings were at the correct stage after 2 days (before the developing root tips became too small). Whole seedlings were placed in 0.002 M 8 hydroxyquinoline at 11.00, and left overnight at 4°C, before fixation and storage in 3:1 absolute ethanol:glacial acetic acid. After fixation the roots were treated in 5 M HCl for 10 min at room temperature, the acid being replaced by 70% ethanol. Then the meristematic region was cut off, and placed on a cleaned microscope slide in a drop of 2% orcein (Sigma certified, Sigma Chemical Company, St. Louis) in 45% acetic acid and the meristematic tissue teased out into the drop of stain using fine tungsten needles. Slides were then heated gently, the tissue was carefully squashed and examined under a Zeiss Large Research microscope using the Planapo 63× objective. Careful drawings were made in order to determine the chromosome count, and suitable preparations were recorded photographically using a Nikon Coolpix digital camera.

Nuclear DNA amounts

For each nuclear DNA amount measurement, approximately 3 cm^2 of freshly harvested leaves of *Salsola* species and hybrids grown in a glasshouse were processed together with the equivalent amount of freshly harvested leaves of *Hordeum vulgare* cv. Sultan as an internal standard (2C = 11.12 pg DNA, seeds obtained from Kew Gardens; Johnston et al. 1999). We also used *Delairea odorata* leaves as an

internal standard for some runs since it had a smaller, but not overlapping, nuclear DNA amount as the *Salsola* samples (*D. odorata* 2C = 6.75 pg DNA, calibrated with *H. vulgare*). The nuclei were isolated using a modified method by Galbraith et al. (1983) and stained for 1 h in the dark with buffer containing 75 mg ml^{-1} propidium iodide (Sigma, St. Louis, MO). A laser flow cytometer (FACSCalibur; Becton-Dickinson, San Jose, CA) was used to estimate the 2C nuclear DNA amount of each sample, run several times over the next hour. Coefficients of variation were typically under 2%.

Results

Microsatellites

Each taxon had a distinctive pattern of microsatellite alleles. *S. tragus*, *S. kali* ssp. *austroafricana*, and *S. paulsenii* contained eight, six, and one species-specific allele, respectively that we used to examine affiliations in Type C and *S.* lax. Type C contained a combination of *S. tragus* and *S. kali* ssp. *austroafricana* alleles that suggested a F1-type hybrid between the two species (Table 1). *Salsola* lax contained alleles from *S. tragus*, *S. paulsenii*, and *S. kali* ssp. *austroafricana.* However, the patterns were not additive for any combination of species. Additionally, there were two unique alleles. These findings suggest that lax is genetically distinct from the three other taxa and, while it is not an F1 hybrid between any of them, it may be a complex hybrid involving three or more species (Table 1).

Table 1 Microsatellite loci and alleles found among the *Salsola* taxa

	BMB 03					CT 04				SB07				SB 09		SB 15			
	228	232	234	248	251	262	138	140	163	184	202	152	168	254	255	271	112	113	141	144	148	159	161
S. tragus			1	1	1		1	1	1		1	1			1	1		1	1		1	1	
S. k . ssp. *austroafricanus*	1	1					1			1				1	1		1						1
S. paulsenii					1		1					1				1		1		1			
Type C		1	1		1		1	1		1	1	1		1	1		1	1			1		1
S. lax	1					**1**	1						**1**	1		1	1	1		1		1	

'1' indicates the allele was present in one or more plants within that taxon. *Bolded values* are alleles unique to *S.* lax

ISSRs and RAPDs

Each individual was genetically phenotyped for 25 RAPD and 43 ISSR (= 68 bands). Genetic distance between individuals was calculated as Euclidean distance. The relationships among individuals were portrayed using UPGMA clustering (Fig. 1). Each morphologically discrete group was also genetically distinct.

We used species-specific bands to examine affiliations in Type C and *S.* lax; *S. tragus* had three bands, *S. kali* ssp. *austroafricana* had nine bands, and *S. paulsenii* had 12 species-specific bands (Table 2). Type C contained fragments specific to *S. tragus* and

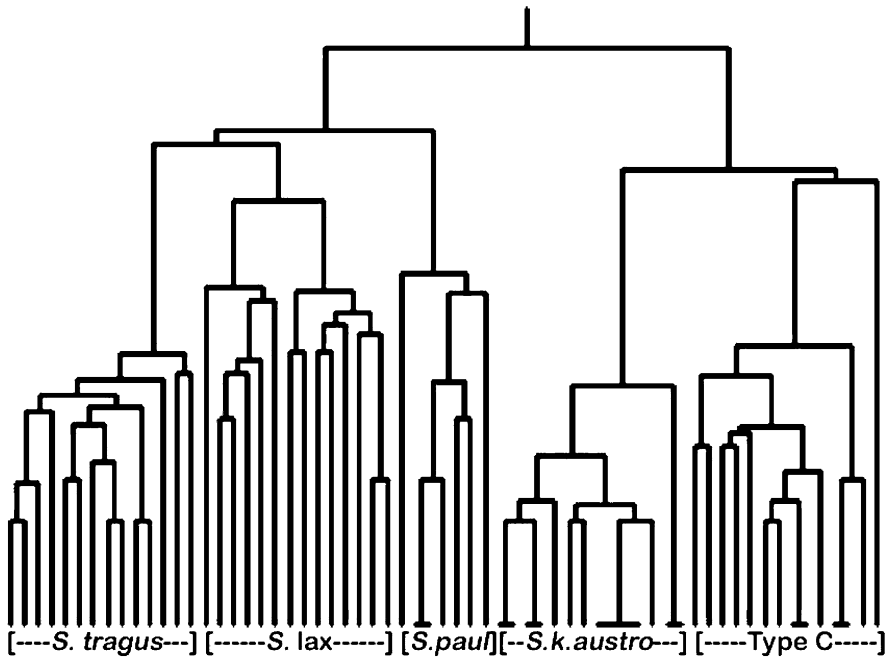

Fig. 1 Dendrogram (UPGMA) of individuals of *Salsola* taxa clustered according to genetic distance based on 68 ISSR and RAPD DNA fragments. Clusters are labeled according to morphologically characterized taxa: *S. tragus*, *S. kali* ssp. *austroafricana*, *S. paulsenii*, *S.* lax, and Type C

S. kali ssp. *austroafricana,* in an additive pattern, and did not contain *any* of the 12 *S. paulsenii*-specific or the two *S.* lax-specific bands, nor any unique bands (Table 2). These patterns are consistent with a F1-type interspecific hybrid between *S. tragus* and *S. kali* ssp. *austroafricana* only. Further, Type C was composed of multiple genotypes (Fig. 1), suggesting multiple hybridizations among parental plants containing different complements of *S. tragus* and *S. kali* ssp. *austroafricana* markers or random assortment within the genomes of each parental complement producing variable progeny or both. *Salsola* lax was highly variable and all individuals contained at least one fragment specific to *S. tragus*, *S. kali* ssp. *austroafricana*, and *S. paulsenii*, in a non-additive pattern for any two species, and most had two unique fragments. These data corroborate the microsatellite results that suggest that *S.* lax is a complex hybrid involving all three species and perhaps an additional species.

DNA sequences

In Fig. 2, we show the parental genome combinations for *S.* lax hybrids. Of the 11 *S.* lax specimens sequenced, ten were genotype 1/14, and one was genotype 2/5/14. The number of copies of haplotypes in each plant is not clear from DNA sequencing. Ploidy level information indicates that lax samples are hexaploid, so it is possible that what appears to be genotype 1/14 is actually genotype 1/1/14 or 1/14/14 or even 1/7/14. In the case of genotype 2/5/14 it is clear that the plant has a higher ploidy level and contains three distinct haplotypes. Earlier samples of *S. paulsenii* ($n = 2$) were both determined to be genotype 1/7 (Gaskin et al. 2006). Sequencing of additional plants is needed to determine if haplotype 14 is from the poorly sampled *S. paulsenii* or another species. It does not seem likely that haplotype 14 is from *S. tragus* or *S. kali* ssp. *austroafricana*, as it did not appear in any of the 88 sequenced plants identified as those two species in Gaskin et al. (2006). Since *S. tragus* and *S. paulsenii* share haplotype 1, it appears from DNA sequence data that *S.* lax plants are hybrid combinations of either *S. tragus* or *S. paulsenii* and an as of yet unidentified source of haplotype 14.

Chromosome counts and nuclear DNA amounts

2C nuclear DNA amounts of *S. tragus* (based on plants from six populations) and of *S. kali* ssp. *austroafricanus* (based on plants from three populations) are 3.15 ± 0.02 (SE) and 2.70 ± 0.04 (SE) pg of DNA,

Table 2 Frequencies of species-specific ISSR/RAPD bands

Taxa	*S. tragus*	*S.k.austroafricanus*	Type C	*S.* lax	*S. paulsenii*
Number of samples	14	14	13	14	8
Number of species-specific bands	3	9	0	2	12
Primer_band		Band Frequency			
835_600	1.00	0	0.92	0.64	0
835_730	0.29	0	0.00	0.64	0
890_700	1.00	0	1.00	1.00	0
A8_580	0	0.14	0.00	0.57	0
A8_720	0	1.00	0.77	0.00	0
A18_650	0	1.00	1.00	0.57	0
D20-325	0	1.00	1.00	0.93	0
810_770	0	1.00	1.00	0.00	0
835_850	0	1.00	1.00	0.29	0
840_1400	0	0.86	1.00	0.00	0
840_1600	0	1.00	1.00	0.50	0
841_1300	0	1.00	0.92	0.00	0
C18_850	0	0	0	0.64	0.88
A18_750	0	0	0	0.64	0.63
H2_870	0	0	0	0.64	0.88
812_900	0	0	0	0.71	0.88
825_800	0	0	0	0.00	0.75
826_830	0	0	0	0.71	0.25
835_620	0	0	0	0.00	1.00
835_1400	0	0	0	0.64	1.00
840_1000	0	0	0	0.57	0.75
850_1100	0	0	0	0.76	0.88
890_950	0	0	0	0.86	0.88
890_1050	0	0	0	0.00	0.88
H8_590	0	0	0	0.79	0
A18_980	0	0	0	0.79	0

Bands specific to each species are outlined (double lines for *S. tragus*, bold line for *S. kali* spp. *austroafricana*, single line for *S. paulsenii*), bands specific to taxon *S.* lax are shaded in grey

respectively, corresponding to chromosome counts of $2n = 36$, and $2n = 18$, respectively (Table 3; Fig. 3). These are the first C-values reported for *Salsola*, and they fit comfortably alongside other values for the family (Bennett and Leitch 2005). No nuclear DNA amount was measured for *S. paulsenii* ($2n = 36$) because we were not able to grow it successfully in the glasshouse. All the nuclear DNA amounts of the *Salsola* hybrids are much greater than that of *S. tragus* and *S. kali* ssp. *austroafricanus*. For three Type C individuals, we found variable nuclear DNA amounts, $2C = 4.50$ pg, and $2C = 4.08$ pg, corresponding to hexaploid plants ($2n = 54$) from two populations and a single aneuploid plant ($2n = 42$ or 43), respectively. *Salsola* lax, represented by one individual, had a nuclear DNA amount of $2C = 4.91$ pg and also had $2n = 54$ (Table 3).

As may be seen from Fig. 3 we are working with a series of taxa with numerous small (mostly under 2 μm) chromosomes. This means that only the most highly condensed mitoses can be used for counting. A consequence of this is that any potential size differences are minimized. Figure 3b shows two cells with different degrees of chromosome condensation. The chromosome squashes do not show any obvious difference in chromosome size between any of the taxa, but under the circumstances such a difference would be difficult to discern.

Discussion

Polyploidy is a widespread evolutionary phenomena in plants. As much as 70% of all angiosperm species have undergone chromosome multiplication (autopolyploidy) or interspecific hybridization and chromosome duplication (allopolyploidy) that have given rise to new species (Masterson 1994). This estimate is

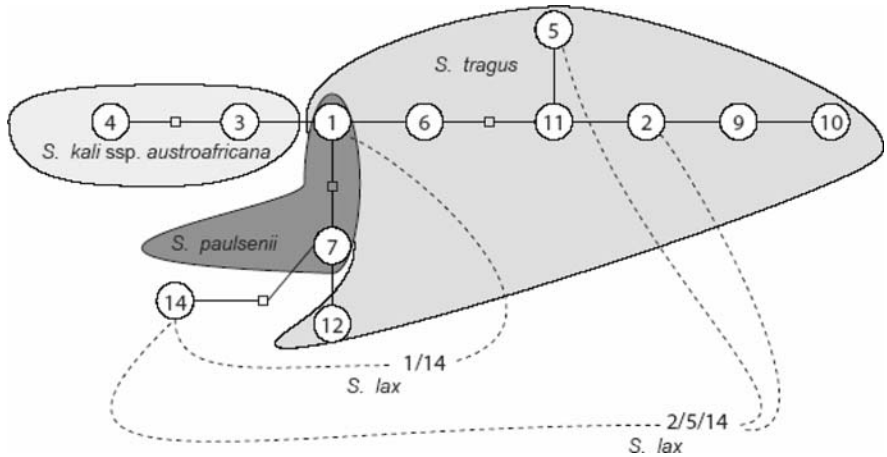

Fig. 2 Haplotype network of DNA sequences of the fourth intron of the PEPC gene region for *Salsola* species. *Circles* represent haplotypes recovered, and *squares* along lineages in between *circles* indicate haplotypes not recovered. Each link between haplotypes indicates one mutational event. *Dotted lines* indicate how haplotypes may have been combined to form *S.* lax genotypes 1/14 and 2/5/14. The loops that surround portions of the haplotype network indicate taxonomic status of plant collections sampled

Table 3 Chromosome numbers, 2C nuclear DNA content, and assumed ploidy level of *Salsola* taxa examined in this study

Taxon	Nuclear DNA content (pg ± SE)	Chromosome number	Ploidy
S. paulsenii	n. d.	36	4X
S. tragus	3.15 ± 0.02	36	4X
S. kali ssp. *austroafricanus*	2.70 ± 0.04	18	2X
S. lax	4.91 ± 0.03	54	6X
Type C	4.50 ± 0.04	54	6X
Type C (aneuploid)	4.08 ± 0.02	42, 43	4X + 6, 7

expected to increase as more sophisticated molecular methods are used to uncover ancient polyploidy speciation events, so-called paleopolyploids (Levy and Feldman 2002). Polyploid plants are thought to have many advantages over diploid plants such as having much broader phenotypes to tolerate more variable environments, as well as having multiple copies of genes, some of which may evolve to have new functions (Comai 2005). On the other hand, polyploidization is also typically associated with whole genome responses to reduce the conflicts of multiple copies of some genetic material and/or to reduce the costs associated with maintaining and replicating large amounts of DNA (Leitch and Bennett 2004). These responses include epigenetic changes such as broad methylation patterns to silence parts of the genomes such as is seen in *Spartina* hybrids (Ainouche et al. 2004; Salmon et al. 2005), as well as chromosomal alterations such as deletions (and rarely, insertions) of coding and non-coding DNA (reviewed in Leitch and Bennett 2004). These whole genome responses can occur fairly rapidly, often times within a few generations (Han et al. 2005; Skalická et al. 2005).

Five new allopolyploid plant species have been discovered within the last 110 years (Abbott and Lowe 2004); four of these species involve one or two introduced species interacting with native plants of the same genus. Here we report the determination of new allopolyploid species in *Salsola* in California. Type C ($2n = 54$), an allohexaploid species resulted from hybrid formation between exotic species *S. tragus* ($2n = 36$) and *S. kali* ssp. *austroafricana* ($2n = 18$). A second allopolyploid species, *S.* lax ($2n = 54$), contained alleles and DNA fragments specific to all three recognized species and unique markers as well and may be a multi-species complex. Development of a

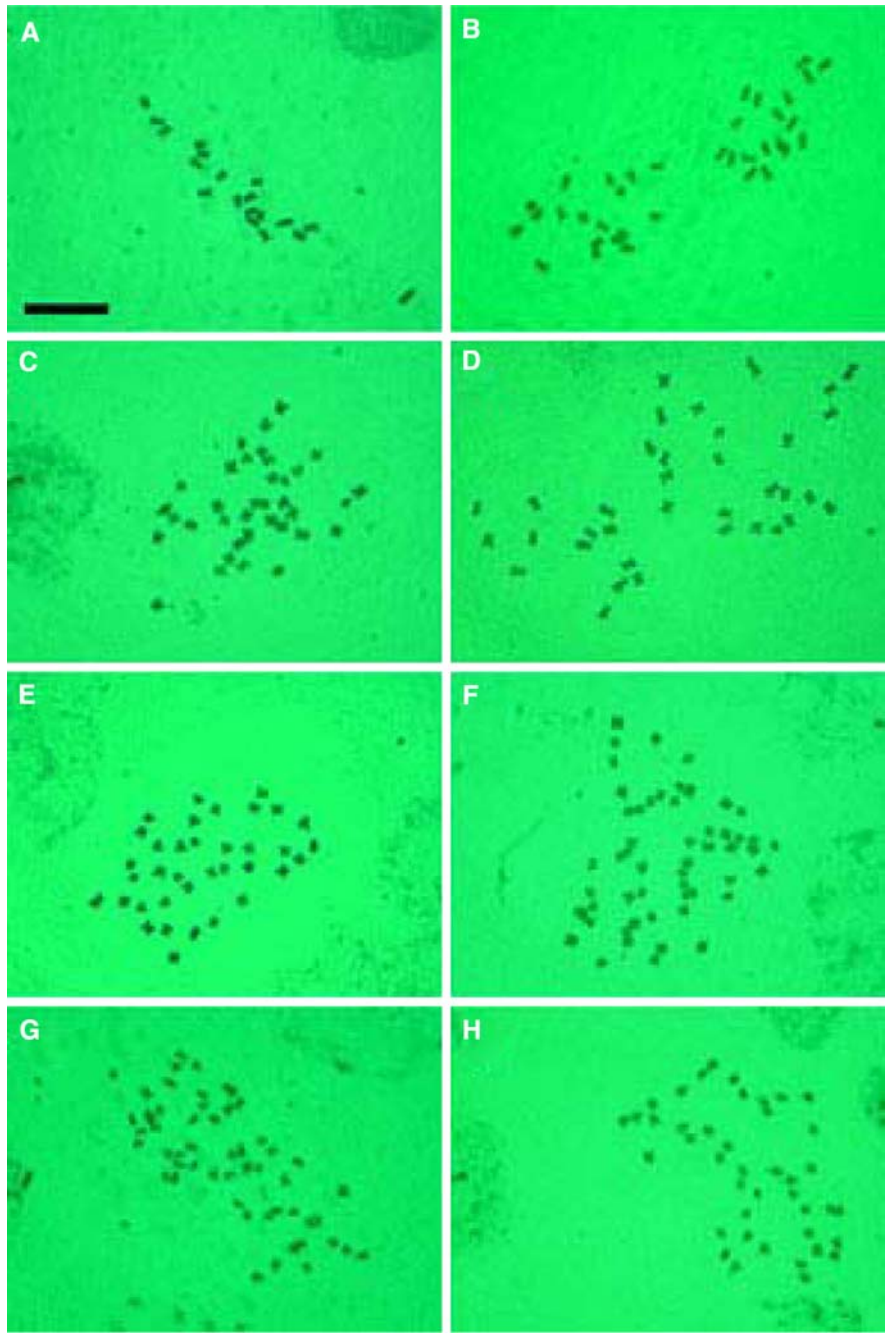

Fig. 3 Cytology figure legend. **a** *S. kali* ssp. *austroafricana* Arizona, $2n = 18$, **b** *S. kali* ssp. *austroafricana* Arizona, $2n = 18$—note two cells with different degrees of contraction, **c** *S. paulsenii*, $2n = 36$, **d** *S. tragus*, $2n = 36$, **e** *S. tragus*, $2n = 36$, **f** *S.* lax, $2n = 54$, **g** Type C, $2n = 54$, **h** Type C, $2n = 42$ aneuploid. Scale bar = 10 μm

robust set of microsatellite and ISSR/RAPD markers made the analysis of hybrids straightforward.

The *Salsola* Type C allopolyploid

The allopolyploid species, Type C, has been found in two discrete populations about 100 miles apart in California's southern Central Valley. Type C plants differed from one another at several ISSR/RAPD sites where the parental species exhibited polymorphism providing genetic evidence of multiple origins. Further genetic evidence of multiple origins was provided by DNA sequence analysis from a previous study (Hrusa and Gaskin, in review) that revealed

three distinct Type C haplotypes. Microsatellite alleles and DNA fragments exhibit an additive pattern of genetic traits unique to *S. tragus* and *S. kali* ssp. *austroafricana* that is consistent with a F1 hybrid. A chromosome number of 54 for two out of three Type C plants, one from each population, implies chromosomal doubling of a F1 hybrid with 27 chromosomes to 54 chromosomes to form an allohexaploid. Type C has a 2C nuclear DNA amount that is greater than either of its parent species, *S. tragus* (2C = 3.15 pg) and *S. kali* ssp. *austroafricanus* (2C = 2.70 pg). Following an additive model of hybridization as was seen in allopolyploid *Dactylorhiza* (Aagaard et al. 2005), *Tragopogon mirus* (Pires et al. 2004), and *Spartina* (Ayres et al. 2008), one would expect a nuclear DNA amount of 2C = 5.85 pg for Type C. Here, we found that the 2C nuclear DNA amount of Type C is substantially less, only 4.50 pg, representing an approximately 23% reduction. Somewhat similar reductions in genome size (ca. 15%) were found for the allopolyploid *Tragopogon miscellus* (Pires et al. 2004) and natural *Paspalum* allopolyploids (Vaio et al. 2007). This is likely the result of genome downsizing, a reduction in genome size that is sometimes found in polyploid plants (reviewed by Leitch and Bennett 2004).

There are two hypotheses to explain the presence of Type C in California—the species formed somewhere else where the two parental species overlap and it was subsequently introduced into California, or Type C has formed in California via interspecific hybridization and allopolyploidization in the ca. 100 years following the arrival of *Salsola* species in the Central Valley (Shinn 1895). Since the detection of *S. kali* ssp. *austroafricana* using genetic markers <10 years ago (Ryan and Ayres 2000), plant taxonomists throughout the world have re-examined their herbarium collections of *Salsola* section *Kali* in an attempt to find the native range for this species, to no avail. Only recently was this species identified using DNA sequence data as *S. kali* ssp. *austroafricana* from South African plants (Gaskin et al. 2006) and identified based on fruit morphology as a widespread pest plant of long standing (before the European invasion circa 1770) in Australia (Borger 2007) where it had been misidentified as *S. tragus* (Rilke 1999). While there is no agreement on whether *S. kali* ssp. *austroafricana* is native to South Africa or Australia, there has been no report of *S. kali* ssp. *austroafricana* within the native range of *S. tragus* in Eurasia (Ryan et al. 2007). Large areas of overlap of the two parental species have been documented only in California (Akers et al. 2002). Further, if Type C was introduced with either of its parental species, both of which are widespread in the American West, Type C's distribution should be continuous and widespread rather than relatively restricted to two disjunct populations separated by 100 miles. These observations support the hypothesis of at least two recent speciation events in California.

The *S.* lax hybrid complex

Although hybridization has long been suspected between *S. tragus* and *S. paulsenii*, both in the US (Arnold 1972) and in their native range (Rilke 1999), naturally occurring hybrids have never been verified using molecular genetics. DNA sequence analysis confirmed that one plant of *S.* lax, a putative *S. paulsenii* × *tragus* hybrid, contained a haplotype from both *S. paulsenii* and *S. tragus*; no conclusions could be made regarding the other 10 plants which contained a haplotype from an unidentified source. Microsatellite and ISSR/RAPD fragments showed that the *S.* lax plants were not F1 hybrids between *S. paulsenii* and *tragus* as species-specific genetic traits were not additive. A chromosome number of 54 suggested that *S.* lax is not an introgressant hybrid between these two species as we would expect introgressant hybrids to have the same chromosome number as the parents, i.e., $2n = 36$. Further, we found alleles and DNA fragments unique to *S. kali* spp. *austroafricanus*, as well as alleles and fragments unique to *S.* lax, none of which were ubiquitous in all individuals of *S.* lax. Taken together, we interpret the foregoing to mean that *S. kali* spp. *austroafricanus* did NOT recently hybridize with introgressant *S. paulsenii* × *tragus* hybrids in a manner analogous to the formation of Type C. If this were the case, we would expect to find an additive pattern of *S. kali* spp. *austroafricanus* genetic traits in *S.* lax, which we did not. Additionally, we have not found the source of the genetic traits unique to *S.* lax. It is possible that more intensive sampling, especially of *S. paulsenii*, would reveal the contributing species. It is also possible that another *Salsola* species has contributed a part of its genome to this taxon. The origin and ancestry of

S. lax remains a mystery that awaits resolution through additional nuclear and chromosomal DNA sequence analysis, and karyological assessments of *S.* lax plants from California and the US Great Basin, and suspected *S. paulsenii* × *tragus* hybrids from the native range.

Invasiveness of *Salsola* hybrids

After the introduction of *S. tragus* to North America, its range increased immediately as it moved into available niches of poorly managed agricultural and range lands. There is no evidence of the prolonged lag phase, up to 100 years, summarized by Ellstrand and Schierenbeck (2000) in cases of intra- or interspecific hybridization leading to micro-evolutionary development of more aggressive weed species. However, with the recent establishment of at least three distinct species of *Salsola* in California, the possibility of evolution through hybridization and polyploid formation is apparently being realized.

Documentation of hybrid taxa in *Salsola* populations does not in itself demonstrate that hybrids are or will be more invasive than the parental species. First, taxonomic uncertainty and formerly cryptic species like *S. kali* spp. *austroafricanus* make it difficult to reconstruct invasion histories to determine the invasion dynamics of the parental species. Second, *S.* lax and Type C are only herein identified as distinct genetic entities, and their spread has not been fully mapped through detailed surveys. Finally, we have not ascertained the recent or phylogenetic lineages of *S.* lax. Is the taxon we examined in this study the same one described by Arnold (1972) as occurring in Utah? How is it related to the purported *S. tragus* × *paulsenii* hybrids described by Rilke (1999) from both the introduced and native ranges? The *S.* lax taxon may be a mixed bag of species and hybrids, or a stabilized allopolyploid of more ancient lineage. One approach to study the potential invasion ability of the hybrid taxa relative to the three named species is common garden studies. Fitness in common environments as assessed by survival, growth, and seed and pollen production and viability, and ecological tolerance studies in which arrays of plants are grown in soils of increasing salinity (Rilke and Reimann 1996), for example, are ways to compare fitness and test physiological tolerance and plasticity for environmentally relevant abiotic stress. Salmon et al. (2005) suggested that the differential methylation patterns among allopolyploids may be responsible for the greater environmental tolerance and greater morphological plasticity than the parental species in *Spartina* allopolypoids, for example.

Control of *Salsola* species

Salsola tragus infests ca. 41 million hectares in the western United States (Smith 2005). Tillage and herbicides are used on a limited basis to control tumbleweed populations in agricultural settings and along roadsides. In California, $1.2 million are spent annually to remove the plants from along major highways where, if left untreated, they become hazards as they tumble freely into traffic.

Resistance to chlorsulfuron, an herbicide that inhibits acetolactate synthase (ALS) has been demonstrated in Russian thistle in Canada, Washington, Montana, Idaho, Oregon, and California (summarized on (http://www.weedscience.org/Case/Case.asp?ResistID=411) dating from 1987. Resistant plants have been found on over 1,500 sites. Cross resistance to two different herbicides that act to inhibit ALS (chlorsulfuron and sulfometuron) has been found in a California biotype (http://www.cdfa.ca.gov/phpps/ipc/weedinfo/salsola.htm#anchor12345). These observations support the conclusion that with or without multiple species and hybrids, the potential for rapid evolution exists within Russian thistle due to selection within naturally occurring genetic variation. Biological agents may be a good approach to control of a widespread weed that is developing herbicide resistance.

There are no native species or agricultural commodities of *Salsola* in North America so the danger of a biological control agent escaping to attack valuable crop or native species is minimal. However, different responses by insect herbivores and fungal disease pathogens being considered for biological control agents for various *Salsola* taxa may reflect variations in specificity among pure species and hybrids, particularly if the biocontrol agents were selected originally to target one parental species. *S. tragus* and *S. kali* ssp. *austroafricana* were differentially susceptible to potential biological control agents, a fungal pathogen, *Colletotrichum gloeosporoides*, and a gall-forming midge (*Desertovellum stackelbergi*) (Bruckart et al. 2004; Sobhian et al. 2003b). On the other hand, the eriophyid mite, *Aceria salsolae*, collected on *S. tragus*

in Greece, successfully reproduced on *S. tragus*, *S. kali* ssp. *austroafricana*, Type C, *S.* lax, *S. paulsenii*, and *S. collina*, all of which are considered noxious weeds in *Salsola* section *Kali*, and reduced the size of *S. tragus* plants by 66% under artificial conditions (Smith 2005). Future work on *Salsola* biological control clearly must take into account both the source of the agent, and its usefulness against the array of taxa it will face in the American West.

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